jz-2022-00896b.R1

Name: Peer Review Information for "Measuring Photophysical Transition Rates with Fluorescence Correlation Spectroscopy and Antibunching"

First Round of Reviewer Comments

Reviewer: 1

Comments to the Author

The manuscript titled "Measuring photophysical transition rates with fluorescence correlation spectroscopy and antibunching" by Sakhapov et al. presents a novel approach to estimate the rates of populating (inter-system crossing, ISC) and de-populating (phosphorescence, PH) the triplet excited state in a three-level system with S0, S1 & T1 states, from nanosecond FCS (nsFCS) and from the microsecond-part of FCS curves. While the triplet-blinking (a.k.a. photophysics) of fluorescent dyes have been reported from FCS measurements as relaxation times, they are an outcome of contributions from both the ISC & PH processes, and their values are not always known. This manuscript provides simple means for estimating the values of the rate constants for these processes. The development presented here is important for researchers who are using fluorescence-based techniques, and have to account for all the intertwining processes, including the dye triplet-blinking. Altogether, the work summarized in the manuscript presents an important advancement, and I am happy I had the chance to review it.

I do have comments, I hope can help improve the manuscript even more.

Major comments:

1. Time separation between antibunching, triplet blinking and diffusional processes: the authors assume the antibunching, triplet blinking and diffusional processes are well-separated in time, and therefore can be taken as a multiplication of three independent processes. While this is a sound assumption for the time separation between the antibunching and the triplet blinking processes, it might not always hold between the triplet blinking and diffusional processes. Since the change in the autocorrelation due to diffusion is not described by an exponential decay, the short diffusional timescales many times overlap with the long tail of the exponential process governing the triplet-state photophysics. I suggest the authors add a few lines explaining how the data should be treated in such cases.

2. When the authors define the autocorrelation as equivalent to the probability of detecting a second photon at time t+t0, after a first one has been detected at time t0, they take into account the theory they developed for the probability of surviving in S1 as if any depletion back to S0 ground state leads to a photon emission. The theory is using the fluorescence lifetime, which is really the reciprocal of the sum of the radiative and non-radiative processes. However, only the radiative S1->S0 transition leads to the emission of a photon. The theory basically assumes that the any S1->S0 transition ends with a photon, whether at time t0 or at time t0+t – which happens only if the radiative quantum yield of the S1->S0 processes is 1. This is well expressed especially in the sentence: " On a short timescale, this s1(t)

is given by eq. (2), and on a longer timescale, it is given by the product of the probability s(t) to find the molecule in one of its singlet states, see eq. (5), times the probability that it is then in its excited state, see eq. (3)". In my view, the theory should be expanded to take the fluorescence quantum yield also into account, when relating the theory to actual fluorescence photons that are detected.

3. In the theoretical part, when the authors explain the different contributions to the microsecond photophysical dynamics, they show that if the excitation rate is low, the dominant process between the two main processes is the T1->S0 process, and that "Whereas the result for k_ph is relatively robust (it is the intercept of the curve k_ph + <kappa>k_isc with the abscissa in the limit of zero excitation intensity), the value of k_isc crucially depends on the correct estimate of <kappa>". As I recall from the development of nsFCS by Schuler and co-workers, nsFCS is performed at low excitation power, exactly so that the relaxation will be governed mostly by the T1->S0 transition, and hence k_ph can be inferred directly from the microsecond part of FCS curves.

4. The numerical simulations were performed using the Gaussian approximation of light intensity distribution in space. While this approximation is very useful, I wonder how the simulation results will look like after using a more realistic approximation, for instance one that can be constructed numerically using PSFlab.

5. The numerical calculation of the autocorrelation functions was performed on the whole continuum in space and time. Then, deviations of the fitted parameter values from the ground truth ones are reported in Fig. 3. I have two comments regarding this procedure:

a. I take it that the error is taken is the deviation between the ground truth values and the values from the linear fit, hence are dependent only on the linear fit performance. However, the linear fit was performed on smooth analytical functions. A Monte Carlo simulation of G_a and G_p would introduce additional errors which could then be taken as realistic relative errors.

b. At the timescales of G_a & G_p, SPAD afterpulsing should also be taken into account.

6. Regarding the analyses of the experimental results, the authors perform the experiments as a function of laser powers, and the laser powers used are quite high values, including at 1 mW! These are very high powers, expected to introduce saturation effects, which would end with more than the three-state system in the presented theory. In fact, this resulted in the expected deviation for the high power measurements of ATTO 655. I believe it could be useful for the critical reader to present the excitation power versus emission rate curve of each dye they measured at different laser powers to show the last laser power is beyond the saturation limit with the traditional plots that are used.

7. Regarding the theoretical description, the authors describe in eq. 1 the rate equation for populating and depopulating the S1 excited-state. The authors take k_exc as the rate of populating the S1 state and then K_exc+(1/tau) as the rate of depopulating state S1. From that I presume the authors take into account the possibility of depletion from the excited state to the ground state due to the excitation laser, thus via stimulated emission. However if this is the case:

a. It assumes the electron stays at that vibronic state in S1 for more than a few ps before vibrational relaxation (VR) brings it to lower vibronic states in S1. The ps survival at that vibronic state before VR occurs is negligible relative to the S1 lifetime, and can be neglected. The excitation rate k_exc can be

taken as the net successful pumping of the electron from S0 to S1, with an efficient VR process within S1 vibronic states.

b. Why not include also the possibility for S1->S2 transitions? If the excitation power can be so strong as to induce enough immediate depletions from S1 to S0, then why not also consider S1->S2?

8. Regarding the assumption that the time separation between the S processes and the S to T transitions allows treating the S0->S1 and S1->S0 similarly within the S equilibrium, stated in the sentence: "Next, let us consider the slow kinetics of intersystem crossing and phosphorescence, and let us assume that this takes place on such a slow time scale that at any moment in time, the fast transitions between S0 and S1 are in equilibrium":

a. If the excitation rate is low (e.g., low laser power), then the residence time in the ground state is high, and can perhaps be comparable to the lifetimes in the triplet state. In this case, the approximation will not hold.

b. Even with this approximation, regardless, the input towards T1 is solely from S1, and so the survival probability in all singlet states is irrelevant, only that in S1.

Minor Comments:

1. The opening sentence in the introduction reads: "Fluorescent dyes have become indispensable for a myriad of microscopy and spectroscopy applications in the life sciences". Please cite a review or two.

2. In the introduction, before the paragraph that starts with "In the present paper, we combine fluorescence antibunching measurements with FCS…", perhaps this is the place to add a few sentences on the antibunching FCS experiments known as nsFCS, presented as later developments by Schuler and co-workers?

3. In eq. 2, the coefficient (k_exc+(1/tau)) is used. Why not replace it with the k_exc-dependent coefficient k_exc / kappa? This is just a suggestion.

4. In the theoretical part, the following is written: "What has been done so far in the literature is to measure and fit correlation curves for different excitation intensities…". Please add references to the literature.

5. The text in the theoretical part reads: "In the next section, we will check by numerical simulation what the expected bias and accuracy of this approach is, in the light that eqs. (11) and (12) are only rough approximations of the exact equations (8) and (7)." However, it should read: " In the next section, we will check by numerical simulation what the expected bias and accuracy of this approach is, in the light that eqs. (11) and (12) are only rough approximations of the exact equations (8) and (9).".

6. In the text, the extinction coefficient is defined using sigma. In the figure it is referred to as epsilon. In practice the typical symbol for the extinction coefficient is epsilon. Additionally, the units of the molar extinction coefficient are M^-1cm^-1, not l M^-1 cm^-1.

7. In eq. 17, the symbol P is not explained. I assume this is the laser power in the back aperture of the objective lens. Please add the description.

8. In the results, the authors write: "Fig. 3(a) shows the model results for the antibunching curves, and fig. 3(b) for the corresponding FCS curves". If the full autocorrelation function is a result of multiplying G_a with G_p, why not also show the multiplication result?

9. The authors write: "… fluorescence lifetime value tau = 4.0 ns which in perfect agreement with reported values". Please cite the literature referred to.

10. The authors report that: "A linear fit was performed on a randomly chosen set of four points, one from each point cloud". I think it would be more robust to represent the cloud of each measurement as a 2D histogram, and then to fit all 2D histograms using a liner plot, to the most probable values of the distributions.

11. Fig. 4 reports the <kappa> values in each experiment, in the captions within each panel. Please report them with their uncertainties +/- values.

12. Fig. 4b: Were the autocorrelation curves globally fit, with the diffusion autocorrelation taken as a global fitting function? If not, that could help.

13. In the description of the experimental system, the routing of the detectors to the HydraGarp400 as START & STOP signals is not described.

14. I have left comments also on the PDF of the manuscript, including some suggestions regarding English

Again, I would be very happy to see this paper published in the Journal of Physical Chemistry Letters, and am sure it would benefit many in the community.

Sincerely,

Dr. Eitan Lerner

Reviewer: 2

Comments to the Author

Fluorescence correlation spectroscopy (FCS) is an important tool in biophysical and life science research. One application is the characterization of photophysical parameters of fluorescent dyes, such as transition rates into the dark triplet state. The latter are important to characterize performance of the dye as label in life science applications. Unfortunately, the exact determination of triplet-state transition rates has so far been biased by the inhomogeneity of the spatial profile of the fluorescence excitation efficiency over the observation volume of the confocal microscope. Several approaches to approach this limitation have been suggested before, however still introducing remaining approximations or high complexity. Skhapov et al introduce a very elegant and straightforward way to solve this issue, employing simultaneous FCS and antibunching measurements. Thereby, the latter are used to give an accurate estimate of the excitation efficiency. The success of this approach in determining accurate triplet-state transition rates is shown through numerically simulated data and experiments on dyes.

This is an important and excellently performed and described study. I highly recommend publication.

I have only very minor comments, which the authors might want to (but do not necessarily need to) consider before final publication.

- Page 2, line 47: One could also cite Eggeling et al Anal Chem 70, 2651-2659 (1998) and Mitronova et al Chemistry A European Journal 16, 4477 - 4488 (2010).

- How far do the timescales between singlet and triplet transitions must differ for the decoupling to be allowed? Any estimation?

- The reason for the bias in using a single constant value over the excitation volume becomes specifically biased at high excitation intensity values, where saturation of transitions become a role. Maybe I missed it, but the authors may want to mention saturation more pronouncedly.

- Page 7, bottom: The authors may want to give references to the previous literature here. Also, one could specifically name the different approaches besides the one mentioned here (as done e.g. in refs 9 and 11), i.e. constant intensity value at half the maximum or other values (e.g. ref 5, Eggeling et al Anal Chem 70, 2651-2659 (1998)), approximation of intensity profile by two Gaussian distributions (ref. 6), or exact numerical description of the saturated fluorescence profile (ref. 11).

- How long are the measurement times and how sensitive is this approach on the measurement time?

- Compare the experimental values of the dyes to literature values.

- Page 12, line 1: Why is only the ratio important and why are the considerations valid for all ratios?

- Page 11, line 49: Shouldn't this be intersystem-crossing rates (instead of phosphorescence)?

Reviewer: 3

Comments to the Author

Sakhapov et al. present a combination of the fluorescence antibunching measurements on the nanosecond with fluorescence correlation spectroscopy on the microsecond timescale in a single experiment with sufficiently high time resolution in order to determine the intersystem crossing rate and the triplet state lifetime in an absolute manner. The accuracy of the method is evaluated and it is validated with two well-known fluorescence dyes. The manuscript is well-written and all necessary data and analysis presented in a comprehensive way.

As all methods and publications stemming from the Enderlein group, the work has been done very thouroughly and the methods is an advance to measure ISC rates in a straight-forward absolute manner. That is great!

There is actually only one minor point that I would like to mention:

What I find a bit confusing is that the rate constant for the transition T1->S0 is called k_ph. "ph" implies that the rate describes a pure radiating transition, but also non-radiative ISC has to be considered here. I would suggest to think of another name.

I can strongly recommend publication in the Journal of Physical Chemistry Letters, but kindly ask the authors to consider my (minor) point raised above.

Author's Response to Peer Review Comments:

Dear Prof. Editor,

We have made all changes requested by you, and we have responded to all comments and questions of the referees, see accompanying letter with a detailed explanation of our revision.

With best regards,

Jörg Enderlein

Drittes Physikalisches Institut
Prof. Dr. Jörg Enderlein

Prof. Dr. Jörg Enderlein
Drittes Physikalisches Institut, Friedrich-Hund-Platz 1· 37077 Göttingen

Tel.: +49 (0) 551 – 39 26908
Secretary: +49 (0) 551 – 39 27714
Fax: +49 (0) 551 – 39 27720
Cell phone: +49 (0) 178 – 538 1130
Email: jenderl@gwdg.de
Https://www.joerg-enderlein.de

Prof. Editor
Senior Editor
Journal of Physical Chemistry Letters

Göttingen, May 3rd, 2022

RE: Journal: The Journal of Physical Chemistry Letters
Manuscript ID: jz-2022-00896b
Original Submission Date: 28-Mar-2022
Title: "Measuring Photophysical Transition Rates with Fluorescence Correlation Spectroscopy and Antibunching"
Author(s): Sakhapov, Damir; Gregor, Ingo; Karedla, Narain; Enderlein, Joerg

Dear Prof. Editor,

We have prepared a revised version of our manuscript. We have addressed all the questions and comments of the reviewers, and you will see the detailed answers below. For convenience, we have also uploaded a PDF of our manuscript where all changes/additions are marked in blue.

We hope that our manuscript may now be acceptable for publication.

With best regards,

Detailed responses to reviewers' comments/questions:

Reviewer: 1

Recommendation: This paper is publishable subject to minor revisions noted. Further review is not needed.

Comments:
The manuscript titled "Measuring photophysical transition rates with fluorescence correlation spectroscopy and antibunching" by Sakhapov et al. presents a novel approach to estimate the rates of populating (inter-system crossing, ISC) and de-populating (phosphorescence, PH) the triplet excited state in a three-level system with S0, S1 & T1 states, from nanosecond FCS (nsFCS) and from the microsecond-part of FCS curves. While the triplet-blinking (a.k.a. photophysics) of fluorescent dyes have been reported from FCS measurements as relaxation times, they are an outcome of contributions from both the ISC & PH processes, and their values are not always known. This manuscript provides simple means for estimating the values of the rate constants for these processes. The development presented here is important for researchers who are using fluorescence-based techniques, and have to account for all the intertwining processes, including the dye triplet-blinking. Altogether, the work summarized in the manuscript presents an important advancement, and I am happy I had the chance to review it.
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- We thank the referee for this important question. Unfortunately, there is no simple theoretical solution. In the manuscript, we added the sentence: "Unfortunately, there is no simple theoretical description for the case when the diffusion time, i.e. the time where the diffusion-related part of the autocorrelation functions has fallen off to one-half of its initial value, overlaps with the timescale of the photophysics. In such cases, one should try to extend the diffusion time by using a larger excitation focus and thus detection volume, which shifts the diffusion-related autocorrelation decay to longer times."

2. When the authors define the autocorrelation as equivalent to the probability of detecting a second photon at time t+t0, after a first one has been detected at time t0, they take into account the theory they developed for the probability of surviving in S1 as if any depletion back to S0 ground state leads to a photon emission. The theory is using the fluorescence lifetime, which is really the reciprocal of the sum of the radiative and non-radiative processes. However, only the radiative S1->S0 transition leads to the emission of a photon. The theory basically assumes that the any S1->S0 transition ends with a photon, whether at time t0 or at time t0+t – which happens only if the radiative quantum yield of the S1->S0 processes is 1. This is well expressed especially in the sentence: " On a short timescale, this s1(t) is given by eq. (2), and on a longer timescale, it is given by the product of the probability s(t) to find the molecule in one of its singlet states, see eq. (5), times the probability that it is then in its excited state, see eq. (3)". In my view, the theory should be expanded to take the fluorescence quantum yield also into account, when relating the theory to actual fluorescence photons that are detected.

- We thank the reviewer for this question. The quantum yield does only affect the *absolute amplitude* of the antibunching and FCS curves, but does not have any effect on the *shape*, i.e. temporal behaviour of these curves. Analytically, it enters as a constant factor that determines the brightness of the molecule. The absolute amplitudes of the antibunching and FCS curves do nowhere enter our photophysical analysis. Thus, before equation (7), we have added the remark: "up to some constant factor which depends for example on the fluorescence quantum yield and overall detection efficiency of the measurement system"

3. In the theoretical part, when the authors explain the different contributions to the microsecond photophysical dynamics, they show that if the excitation rate is low, the dominant process between the two main processes is the T1->S0 process, and that "Whereas the result for k_ph is relatively robust (it is the intercept of the curve k_ph + <kappa>k_isc with the abscissa in the limit of zero excitation intensity), the value of k_isc crucially depends on the correct estimate of <kappa>". As I recall from the development of nsFCS by Schuler and co-workers, nsFCS is performed at low excitation power, exactly so that the relaxation will be governed mostly by the T1->S0 transition, and hence k_ph can be inferred directly from the microsecond part of FCS curves.

- The reviewer is absolutely right that at very low excitation powers, the average probability of finding the molecule in S1 is close to zero, and the triplet state dynamics on the microsecond timescale

is dominated by the triplet state lifetime. This is in perfect agreement with our theory and when we write that k_ph is the intercept of the curve k_ph + <kappa>k_isc with the abscissa in the limit of zero excitation intensity, i.e. zero k_isc.

4. The numerical simulations were performed using the Gaussian approximation of light intensity distribution in space. While this approximation is very useful, I wonder how the simulation results will look like after using a more realistic approximation, for instance one that can be constructed numerically using PSFlab.

- We thank the reviewer for the important question. We use a Gauss-Lorentzian form of the focus for our numerical simulations that has been shown to be an excellent approximation for describing the molecular detection function in a confocal volume, see e.g. reference (23) [T. Dertinger et al., ChemPhysChem 2007, 8, 433–443].

5. The numerical calculation of the autocorrelation functions was performed on the whole continuum in space and time. Then, deviations of the fitted parameter values from the ground truth ones are reported in Fig. 3. I have two comments regarding this procedure:
a. I take it that the error is taken is the deviation between the ground truth values and the values from the linear fit, hence are dependent only on the linear fit performance. However, the linear fit was performed on smooth analytical functions. A Monte Carlo simulation of G_a and G_p would introduce additional errors which could then be taken as realistic relative errors.
b. At the timescales of G_a & G_p, SPAD afterpulsing should also be taken into account.

- We apologise to the reviewer for this misunderstanding. The error shown in Figure 3(d) reflect the deviation of the rates as estimated with our model from the actual rates (ground truth) used for calculating the analytical autocorrelation functions (systematic bias). These errors, which are below 4% across the whole range of phosphorescence rates considered, show that despite the highly non-uniform distribution of k_exc across the detection volume, one can robustly estimate the photophysical rates with our model. Of course, the finiteness of an FCS experiment where a finite number of photons is collected will add another (ideally unbiased) error, but to quantify this additional error was not the purpose of the theoretical analysis presented here. Our analysis gives the minimal possible deviation between fitted and actual values. In the FCS experiments reported later, we use a bootstrapping analysis to quantify also this unbiased error due to the finite number of recorded photons.
- The detector afterpulsing and detector dead times do not play a role in our work since we work with the cross-correlations between two SPADs for the analysis. To the Methods section, we added the sentence "Calculation of all antibunching and autocorrelation curves was done by cross-correlating the signals from these two detectors to exclude any dead-time or afterpulsing effects."

6. Regarding the analyses of the experimental results, the authors perform the experiments as a function of laser powers, and the laser powers used are quite high values, including at 1 mW! These are very high powers, expected to introduce saturation effects, which would end with more than the three-state system in the presented theory. In fact, this resulted in the expected deviation for the high power measurements of ATTO 655. I believe it could be useful for the critical reader to present the excitation power versus emission rate curve of each dye they measured at different laser powers to show the last laser power is beyond the saturation limit with the traditional plots that are used.

- We measured the laser power before the objective. Due to aperture effects and reflectivity losses in the microscope and objective optics, it is very difficult to exactly determine the laser light intensity in the sample precisely. That's why researchers have developed and exploited antibunching measurement to determine these intensities, see citation (17) [Mets, Ü.; Widengren, J.; Rigler, R. *Application of the antibunching in dye fluorescence: measuring the excitation rates in solution*. Chemical Physics 1997, 218, 191–198]. To the best of our knowledge, our reported kappa-values ARE the ABSOLUTE estimates of the excitation rates with which molecules are excited, and this is the most important parameter for determining the photophysical rates in our paper. This is basically the one and most important aspect and message of our paper, that by using antibunching, one determines excitation rates in an absolute and calibration-free manner. In short, the reported kappa-values ARE the absolute, calibration-free values for the mean excitation probability of a dye for one laser excitation pulse.
- The referee is fully correct, that at high excitation rates all kinds of higher-order photophysical processes can set in, and this is what we observe for Atto655 at the highest used excitation intensity. Such higher order processes destroy the linearity between kappa and k_ph+k_isc*k_exc as plotted in Fig.4c and Fig.5, so that the linearity of this dependence is a perfect check whether one can still work with the simple three-state model used in our paper or not. Through these experiments and results, we would like to deliver an effective and powerful message to the entire single-molecule community: The simplistic three-state model is sufficient and robust to describe the photophysics of commonly used dye molecules even under optical mild-moderate saturation conditions. To point this out, we added a line in our conclusion section "However, these results emphasize the broad applicability of the simple three-state model for these commonly used dyes even under moderate optical saturation conditions."

7. Regarding the theoretical description, the authors describe in eq. 1 the rate equation for populating and depopulating the S1 excited-state. The authors take k_exc as the rate of populating the S1 state and then K_exc+(1/tau) as the rate of depopulating state S1. From that I presume the authors take into account the possibility of depletion from the excited state to the ground state due to the excitation laser, thus via stimulated emission. However if this is the case:

a. It assumes the electron stays at that vibronic state in S1 for more than a few ps before vibrational relaxation (VR) brings it to lower vibronic states in S1. The ps survival at that vibronic state before VR occurs is negligible relative to the S1 lifetime, and can be neglected. The excitation rate k_exc can be taken as the net successful pumping of the electron from S0 to S1, with an efficient VR process within S1 vibronic states.

b. Why not include also the possibility for S1->S2 transitions? If the excitation power can be so strong as to induce enough immediate depletions from S1 to S0, then why not also consider S1->S2?

- No stimulated depletion is considered here in this work. When considering the excitation wavelengths and our reported excitation rates, one is orders of magnitude away from any efficient stimulated emission. Any stimulated emission or S1->S2 would also lead immediately to a severely nonlinear relationship between kappa and k_ph+k_isc*k_exc in Figs.3 and 4. We would also like to emphasize that our simple 3-state model fits perfectly well the Rhodamin110 and the three low-intensity Atto655 data – any model extension would immediately lead to underfitting and untrustworthy results for the extended set of model parameters.
  Equation (1) relates to the temporal evolution of the population in the two singlet states assuming the total occupancy in both the states is 1. We have amended eq.(1) to make this more clear.
- The model does not make any assumptions on the residence time in the vibronic states. In a standard FCS experiment, the excitation laser is generally chosen at a wavelength close to the excitation maximum of the dye where no emission takes place and with negligible probability for stimulated depletion. Within the laser excitation powers used in this experiment, we can conveniently ignore higher-level excitation probabilities such as S1 -> S2 transitions.

8. Regarding the assumption that the time separation between the S processes and the S to T transitions allows treating the S0->S1 and S1->S0 similarly within the S equilibrium, stated in the sentence: "Next, let us consider the slow kinetics of intersystem crossing and phosphorescence, and let us assume that this takes place on such a slow time scale that at any moment in time, the fast transitions between S0 and S1 are in equilibrium":

a. If the excitation rate is low (e.g., low laser power), then the residence time in the ground state is high, and can perhaps be comparable to the lifetimes in the triplet state. In this case, the approximation will not hold.

- In the case of low excitation rate, the time scale of the S0->S1 and S1->S0 kinetics is completely dominated by the fluorescence lifetime (1/decay rate S1->S0) , whereas the triplet state dynamics is completely dominated by the phosphorescence lifetime (1/decay rate T1->S0). For all known dyes, these numbers are orders of magnitude apart, and our assumption of well-separated time-scales holds perfectly.

b. Even with this approximation, regardless, the input towards T1 is solely from S1, and so the survival probability in all singlet states is irrelevant, only that in S1.

- We agree with the reviewer. Typical excitation powers used in an FCS experiment lead to an excitation rate on the order of 10^6 – 10^7 Hz which is 100 times slower than fluorescence relaxation which is typically on the order of 10^8 – 10^9 Hz. At low excitation powers, as in one of the previous comments by the reviewer, the probability of finding the molecule in the triplet state is very low so that one can directly estimate k_ph from the blinking behaviour of the dye molecules. This is because the probability of the excited molecule undergoing intersystem crossing is given by the ratio of its intersystem crossing rate vs spontaneous decay rate to S0, which is less than 0.1% for most of the dye molecules. But given this rare probability and the fact that phosphorescence rate is also three orders of magnitude slower than the spontaneous decay rate and comparable to the excitation rate, these two equilibria can be still considered separately. From another viewpoint, the transition S1-> S0 is the only transition that is detectable by our instrument. At low excitation rates, the ratio occupancy of S0 and T1 is governed by the ratio $(1/\tau)/k_{isc}$ which is negligible and these two processes can still be treated separately.

Minor Comments:

1. The opening sentence in the introduction reads: "Fluorescent dyes have become indispensable for a myriad of microscopy and spectroscopy applications in the life sciences". Please cite a review or two.

- We have added citations (1-3).

2. In the introduction, before the paragraph that starts with "In the present paper, we combine fluorescence antibunching measurements with FCS…", perhaps this is the place to add a few sentences on the antibunching FCS experiments known as nsFCS, presented as later developments by Schuler and co-workers?

- We have added the sentence “Recently, however, the measurement of fluorescence correlation curves over ~ten orders of magnitude from picosconds to seconds has seen a renaissance and was successfully used for studying rapid conformational dynamics in proteins” and have added citations (18-23).

3. In eq. 2, the coefficient (k_exc+(1/tau)) is used. Why not replace it with the k_exc-dependent coefficient k_exc / kappa? This is just a suggestion.

- We have changed the equation accordingly.

4. In the theoretical part, the following is written: "What has been done so far in the literature is to measure and fit correlation curves for different excitation intensities…". Please add references to the literature.

- We now mention the corresponding citations (8) and (10-16) after the sentence.

5. The text in the theoretical part reads: "In the next section, we will check by numerical simulation what the expected bias and accuracy of this approach is, in the light that eqs. (11) and (12) are only rough approximations of the exact equations (8) and (7)." However, it should read: " In the next section, we will check by numerical simulation what the expected bias and accuracy of this approach is, in the light that eqs. (11) and (12) are only rough approximations of the exact equations (8) and (9).".

- We have corrected this.

6. In the text, the extinction coefficient is defined using sigma. In the figure it is referred to as epsilon. In practice the typical symbol for the extinction coefficient is epsilon. Additionally, the units of the molar extinction coefficient are M^-1cm^-1, not l M^-1 cm^-1.

- We now consistently use epsilon for the extinction coefficient and have corrected the unit. To avoid any confusion, we have changed the symbol for the detection efficiency from epsilon to epsilon_det.

7. In eq. 17, the symbol P is not explained. I assume this is the laser power in the back aperture of the objective lens. Please add the description.

- We have added the explanation.

8. In the results, the authors write: "Fig. 3(a) shows the model results for the antibunching curves, and fig. 3(b) for the corresponding FCS curves". If the full autocorrelation function is a result of multiplying G_a with G_p, why not also show the multiplication result?

- For optimal visibility, the antibunching curves are shown by plots *linear in time*, whereas the microsecond FCS plots are plots *logarithmic* in time. Putting all into one plot will not improve data visibility at all.

9. The authors write: "… fluorescence lifetime value tau = 4.0 ns which in perfect agreement with reported values". Please cite the literature referred to.

- We added citation (32).

10. The authors report that: "A linear fit was performed on a randomly chosen set of four points, one from each point cloud". I think it would be more robust to represent the cloud of each measurement as a 2D histogram, and then to fit all 2D histograms using a liner plot, to the most probable values of the distributions.

- We fully agree that this would be an alternative, but we are also convinced that our presented method works comparably well. The fit quality is excellent, the obtained standard deviations already smaller than the estimated theoretical systematic bias.

11. Fig. 4 reports the <kappa> values in each experiment, in the captions within each panel. Please report them with their uncertainties +/- values.

- We have added these values to the figure captions of Fig.4 and Fig.5.

12. Fig. 4b: Were the autocorrelation curves globally fit, with the diffusion autocorrelation taken as a global fitting function? If not, that could help.

- We did not fit the microsecond FCS curves with a global fit. The fitted diffusion times were similar (~50 µs) and ~25 times longer than the slowest observed photophysical rate, so that the timescale of interest (photophysics) is well-separated from the diffusion timescale. As explained in the manuscript, we estimated the fit uncertainties by a bootstrap analysis, and the resulting uncertainties lead to error margins for the estimated photophysical rate constants that are already much better than the theoretically estimated systematic bias of the method. Thus, using even more sophisticated fit approaches cannot improve the result uncertainties.

13. In the description of the experimental system, the routing of the detectors to the HydraGarp400 as START & STOP signals is not described.

- Modern-day TCSPC electronics do not work with classical START & STOP mode as ancient ones. The HydraHarp 400 has a continuous clock that is synchronized by electric pulses from the laser, and then times the photodiode signals on up to 16 independent channels independently. We changed the corresponding sentence in the Methods section accordingly to: “Detected fluorescence photons were registered with the high-speed timing electronics HydraHarp 400 and SymPhoTime software (PicoQuant GmbH, Berlin, Germany) in time-tagged time-resoled mode, which timed the photons from the two photodiodes separately on independent channels with a common clock that is electronically synchronized with the pulsed laser.”

14. I have left comments also on the PDF of the manuscript, including some suggestions regarding English

- We sincerely thank the reviewer for his heroic work.

Reviewer: 2

Recommendation: This paper is publishable subject to minor revisions noted. Further review is not

needed.

Comments:
Fluorescence correlation spectroscopy (FCS) is an important tool in biophysical and life science research. One application is the characterization of photophysical parameters of fluorescent dyes, such as transition rates into the dark triplet state. The latter are important to characterize performance of the dye as label in life science applications. Unfortunately, the exact determination of triplet-state transition rates has so far been biased by the inhomogeneity of the spatial profile of the fluorescence excitation efficiency over the observation volume of the confocal microscope. Several approaches to approach this limitation have been suggested before, however still introducing remaining approximations or high complexity. Skhapov et al introduce a very elegant and straightforward way to solve this issue, employing simultaneous FCS and anti-bunching measurements. Thereby, the latter are used to give an accurate estimate of the excitation efficiency. The success of this approach in determining accurate triplet-state transition rates is shown through numerically simulated data and experiments on dyes.

This is an important and excellently performed and described study. I highly recommend publication.

I have only very minor comments, which the authors might want to (but do not necessarily need to) consider before final publication.
- Page 2, line 47: One could also cite Eggeling et al Anal Chem 70, 2651-2659 (1998) and Mitronova et al Chemistry A European Journal 16, 4477 - 4488 (2010).

- We have added both citations.

- How far do the timescales between singlet and triplet transitions must differ for the decoupling to be allowed? Any estimation?

- At least an order of magnitude. Fortunately, for all dyes we know of, the difference is ~3 orders of magnitude (nanseconds for S1->S0 versus microseconds for T1->S0).

- The reason for the bias in using a single constant value over the excitation volume becomes specifically biased at high excitation intensity values, where saturation of transitions become a role. Maybe I missed it, but the authors may want to mention saturation more pronouncedly.

- Saturation is fully considered in our model – it is the kappa factor defined in eq. (3), which describes the increasing saturation of the S0<->S1 transition with increasing excitation intensity k_exc.

- Page 7, bottom: The authors may want to give references to the previous literature here. Also, one could specifically name the different approaches besides the one mentioned here (as done e.g. in refs 9 and 11), i.e. constant intensity value at half the maximum or other values (e.g. ref 5, Eggeling et al Anal Chem 70, 2651-2659 (1998)), approximation of intensity profile by two Gaussian distributions (ref. 6), or exact numerical description of the saturated fluorescence profile (ref. 11).

- We added "In the literature, different estimates of $\langle\kappa\rangle$ assuming different excitation light distribution in the focus have been made. Ref. 13 used a constant intensity value at half the maximum. A constant intensity value was also used in ref. 10. Refs. 9 and 11 approximated the light distribution

by the superposition of two Gaussian distributions, while ref. 16 used an exact numerical description of the saturated fluorescence profile."

- How long are the measurement times and how sensitive is this approach on the measurement time?

- The measurement time for all measurements was 4 h, as written in the Methods section. Longer measurement times lead to more collected photons which improves in particular the antibunching curves and fit quality. An exact quantitative analysis of the quality of FCS measurements on measurement time is extremely complicated, because it depends also on dye concentration, focus diameter, extinction coefficient, detection efficiency, and such an analysis goes far beyond the scope of the present paper. We refer the referee to the publication:

  Kask P, Günther R and Axhausen P 1997 Statistical accuracy in fluorescence fluctuation experiments Eur. Biophys. J. 25 163–9

  where the authors try to give at least some quantitative estimates for the accuracy of FCS measurements.

- Compare the experimental values of the dyes to literature values.

- We have added the comparison with the values reported by Blom et al., ref. (14).

- Page 12, line 1: Why is only the ratio important and why are the considerations valid for all ratios?

- The factor kappa only depends on this ratio. We added to the corresponding sentence "(the factor κ only depends on this ratio, see eq. (3))."

- Page 11, line 49: Shouldn't this be intersystem-crossing rates (instead of phosphorescence)?

- We thank the reviewer for pointing out this mistake. We corrected it.

Reviewer: 3

Recommendation: This paper is publishable subject to minor revisions noted. Further review is not needed.

Comments:
Sakhapov et al. present a combination of the fluorescence antibunching measurements on the nanosecond with fluorescence correlation spectroscopy on the microsecond timescale in a single experiment with sufficiently high time resolution in order to determine the intersystem crossing rate and the triplet state lifetime in an absolute manner. The accuracy of the method is evaluated and it is validated with two well-known fluorescence dyes. The manuscript is well-written and all necessary data and analysis presented in a comprehensive way.

As all methods and publications stemming from the Enderlein group, the work has been done very

thouroughly and the methods is an advance to measure ISC rates in a straight-forward absolute manner. That is great!

There is actually only one minor point that I would like to mention:
What I find a bit confusing is that the rate constant for the transition T1->S0 is called k_ph. "ph" implies that the rate describes a pure radiating transition, but also non-radiative ISC has to be considered here. I would suggest to think of another name.

- These are standard terms and the model works independent of their nature, radiative or non-radiative. We block any phosphorescence photons (if any) due to our band pass filters in our experiments. To make this clearer, we added, when introducing the phosphorescence rate: "(inverse lifetime of the triplet state)".

I can strongly recommend publication in the Journal of Physical Chemistry Letters, but kindly ask the authors to consider my (minor) point raised above.